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Effect of *omp10* or *omp19* Deletion on *Brucella abortus* Outer Membrane Properties and Virulence in Mice

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The distinctive properties of *Brucella* outer membrane have been considered to be critical for *Brucella* sp. virulence. Among the outer membrane molecules possibly related to these properties, Omp10 and Omp19 are immunoreactive outer membrane lipoproteins. Moreover, these proteins of *Brucella* could constitute a new family of outer membrane proteins specifically encountered in the family *Rhizobiaceae*. We evaluated the impact of *omp10* or *omp19* deletion on *Brucella abortus* outer membrane properties and virulence in mice. The *omp10* mutant was dramatically attenuated for survival in mice and was defective for growth in minimal medium but was not impaired in intracellular growth in vitro, nor does it display clear modification of the outer membrane properties. Significantly fewer brucellae were recovered from the spleens of mice infected with the *omp19* mutant than from those of mice infected with the parent strain at 4 and 8 weeks postinfection. The *omp19* mutant exhibited an increase in sensitivity to the polycation polymyxin B and to sodium deoxycholate. These results indicate that inactivation of the *omp19* gene alters the outer membrane properties of *B. abortus*.

Brucellae are gram-negative bacteria that cause human disease and significant worldwide economic losses due to infection of livestock. These bacteria are able to multiply within professional and nonprofessional phagocytes, but the exact mechanisms whereby *Brucella* spp. intracellularly parasitize the host are still to be defined (4, 5, 7, 15, 30, 36, 37).

The *Brucella* outer membrane has been proposed to be involved in virulence (i.e., resistance to bactericidal cationic peptides and polycations, permeability to hydrophobic agents, resistance to divalent cation chelators, and poor activation of bactericidal mechanisms by lipopolysaccharide [LPS]) (for a review, see reference 35). Correlation between these properties and specific surface molecules can be studied by genetically engineering mutations in the genes and determining the resultant phenotype.

The analysis of genetically defined rough mutants of *Brucella melitensis* and *Brucella abortus* confirmed the involvement of lipopolysaccharide O side chain in *Brucella* in vivo survival (1, 24, 25, 34, 45, 46). The recently identified BvrR-BvrS two-component regulatory system is involved in the control of outer membrane properties such as resistance to bactericidal polycations and is highly relevant for the virulence of *B. abortus* (40).

The molecular characterization of several *Brucella* outer membrane proteins (Omps) has been reported over the past years. The genes *omp25*, *omp31*, and *omp2b* (which encode the major 25-, 31-, and 36-kDa Omps, respectively) and the genes *pal*, *omp10*, *omp19*, and *omp1* (which encode the 16-, 10-, 19-, and 89-kDa minor Omps, respectively) have been cloned and sequenced (10, 16, 18, 28, 31, 43, 44, 50; Bearden and Ficht, GenBank accession number U51683). Omp2b functions as a porin, and the 16-kDa Omp shows significant similarity to the peptidoglycan-associated lipoproteins Pal of gram-negative bacteria (32, 44). An *omp25* mutant of *B. melitensis* is attenuated in mice and in the natural host (16a and 16b), indicating that Omp25 is critical for the maintenance of a *B. melitensis* infection. The 10- and 19-kDa Omps (Omp10 and Omp19, respectively) are surface-exposed lipoproteins (i.e., covalently linked to fatty acids) expressed in all six *Brucella* species and all their biovars (8, 42, 43). Omp10 and Omp19 share antigenic determinants with bacteria of the family *Rhizobiaceae* (9), and the only homologs present in the current sequence databases are two protein sequences deduced from the *Mesorhizobium loti* genome (27).

Antibody is elicited to Omp10 and Omp19. By using purified recombinant Omp10 and Omp19, a significant antibody response specific for these Omps could be detected in a large fraction of sera from sheep naturally infected by *B. melitensis*. However, there was no serologic response to these recombinant Omps in cattle naturally infected by *B. abortus* (29, 43). Kovach et al. also reported antibodies to Omp19 in infected mice, goats, dogs, and humans (28).

Notwithstanding the conservation of Omp10 and Omp19 among *Brucella* species, their possibly unique association with the *Rhizobiaceae*, and their immunoreactivity, no biological function has been assigned to these Omps. To define the function and the pathogenic significance of Omp10 and Omp19, we constructed deletion mutants of the corresponding genes by allelic replacement. The *omp* mutants were used to examine the role for both Omps in bacterial growth, outer membrane properties, and virulence, as assessed in appropriate in vitro (cell culture) and animal models.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. A variant of *B. abortus* strain 544, CO_2 independent and resistant to nalidixic acid (Nal^r), was obtained from J.-M. Verger, Laboratoire de Pathologie Infectieuse et Immunologie, Institut National de la Recherche Agronomique, Nouzilly, France. *B. melitensis* rough mutant B3B2 results from mini-Tn*5* transposon insertion in the perosamine synthetase gene (25). *Escherichia coli* strain S17-1 (39) was kindly provided by G. Cornelis, Microbial Pathogenesis Unit, Christain de Duve Institute of Cellular Pathology, Brussels, Belgium. Nalidixic acid, kanamycin, ampicillin, and/or chloramphenicol was added when appropriate at the following concentrations: 25, 25, 25, and 30 μg/ml, respectively. *Brucella* organisms were grown on tryptic soy agar supplemented with 0.1% (wt/vol) yeast extract (TSAYE), on 2× YT (2YT) agar (38), in tryptic soy broth with 0.1% yeast extract, or in 2YT broth. A minimal medium slightly modified from that of Gerhardt et al. was used (nitrogen and energy were supplied by glutamic acid, lactic acid, and glycerol) (23). The *Brucella* growth rate in liquid media was monitored by recording a culture's optical density (OD) at 590 nm. For carbon substrate utilization patterns, tests were performed using 96-well GN MicroPlates (Biolog, Hayward, Calif.) mainly as directed by the manufacturer. Briefly, *Brucella* organisms freshly grown on TSAYE were recovered, suspended in 0.85% NaCl, and spectrophotometrically standardized to 2×10^9 cells/ml. These suspensions were then added to individual wells (150 μl) and incubated for 24 h at 37°C in 5% CO_2. Color reactions were determined visually (51).

Construction and characterization of *omp* mutants. In order to allow our constructs to be mobilized from a donor strain expressing RP4-conjugative function, like *E. coli* S17-1, the f1 origin of replication of pBluescript SK(−) (Stratagene, La Jolla, Calif.) was excised by *Ssp*I restriction and replaced by a 0.76-kb filled-in *Sma*I-*Sal*I fragment encoding the RK2 origin of transfer (excised from pTJS82, kindly provided by G. Cornelis). This generated the plasmid pSK-oriT.

The plasmid pD192 was designed for the replacement of the first 320 bp of the *omp19* coding sequence by the kanamycin resistance cassette (*kan*). A 479-bp *Bsm*I-*Nru*I fragment was excised from plasmid p191 (43) and replaced by the filled-in 1.3-kb *Bam*HI *kan* cassette from pUC4K (Pharmacia P-L Biochemicals, Uppsala, Sweden). The entire insert containing 261 bp located upstream of *omp19* and 223 bp of the end of *omp19* separated by the *kan* marker was recovered by *Eco*RI digestion and subcloned into the *Eco*RI site of pSK-oriT, producing plasmid pD192. This plasmid was transferred to *B. abortus* 544 Nal^r by conjugation as described by Verger et al. except that the mating time was shortened to 1 h (49). All the transconjugants isolated resulted from a single crossover in the 5′ flanking region of *omp19* (5′ integrant) or in the 3′ end of *omp19* (3′ integrant), as demonstrated by Southern blot analysis. No *omp19* deletion mutant was isolated using pD192. The use of larger flanking regions should increase recombination frequency and, accordingly, mutation efficacy. In order to isolate a larger DNA fragment containing *omp19*, we took advantage of the pD192 integrant clones.

Genomic DNA was prepared from 5′ and 3′ integrant clones, digested with *Hin*dIII and *Bam*HI, respectively, and ligated. After transformation into *E. coli* XL1-blue and selection on ampicillin plates, plasmid DNA was extracted from some colonies and digested by *Hin*dIII and *Bam*HI, for clones recovered from a 5′ and a 3′ integrant, respectively. Plasmid pIa19.16 was selected, the 1.8-kb insert of which contained in addition to *omp19* about 1 kb of sequence downstream of this coding sequence. Plasmid pIb19.22 was also selected and was characterized by a longer insert containing *omp19* and about 10 kb upstream of this sequence. Both clones expressed Omp19 as shown by immunoblot analysis with anti-Omp19 monoclonal antibody (MAb) A76/02A04/A07. A 2.4-kb *Hin*dIII-*Sma*I fragment of pIb19.22 located immediately upstream of *omp19* was subcloned into pSK-oriT as well as a 1-kb *Bgl*I-*Cla*I fragment of pIa19.16 encompassing the end of *omp19*. Plasmid pD193 was obtained by cloning the *kan* cassette into the *Eco*RI site located between these two segments. In pD193, *omp19* coding sequence is deleted from the first 396 bp.

The plasmid pDS10 was designed for the replacement of part of the *omp10* coding sequence by the *kan* cassette. Two *Brucella* DNA sequences, a 1.62-kb *Eco*RI-*Sma*I fragment and a 1.4-kb *Sac*I-*Eag*I fragment, both excised from p101.2 (43), and located upstream and downstream of *omp10*, respectively, were cloned into the pSK-oriT corresponding sites. The *kan* cassette was inserted as a 1.3-kb *Bam*HI fragment from vector pUC4K between these two elements. In the resulting pD103 construct, the *omp10* coding sequence was deleted from a 266-bp internal fragment. The *sacB* gene of *Bacillus subtilis* along with its regulator *sacR* sequence was excised from plasmid pUCD800 (kindly provided by G. Cornelis) (22) as a 2.6-kb *Bam*HI-*Pst*I fragment and cloned into the corresponding sites of pD103, leading to plasmid pDS10.

The constructs pD193 and pDS10, both unable to replicate in *B. abortus*, were conjugated from *E. coli* S17-1 into *B. abortus* 544 Nal^r. A double crossover due to homologous recombination events in each of the *omp* flanking arms resulted in replacement of the *omp* sequence by the *kan* marker and loss of the delivery vector sequences. *Brucella* transconjugants were selected in the presence of nalidixic acid, kanamycin, and 5% sucrose when needed. Transconjugants were then tested for ampicillin sensitivity to screen for or to confirm (in the case of pDS10 transconjugants) loss of suicide vector sequences.

To provide genetic evidence in the transconjugants of *omp* replacement by the *kan* cassette, DNA isolated from the mutant and wild-type strains was digested with *Hin*dIII and hybridized to *omp*, *kan*, and *sacB* probes. A chromosomal DNA miniprep procedure was performed as described previously (48). An *omp19*-specific probe was prepared from the 0.96-kb *Eco*RI insert from plasmid p191 (43), and an *omp10*-specific probe was prepared from a 1.65-kb *Eco*RI-*Sma*I fragment containing the 5′ end of *omp10* excised from p101.2 (43). A *kan*-specific probe was prepared from the *Bam*HI fragment containing the *kan* cassette from pUC4K, and a *sacB*-specific probe was prepared from a 2.6-kb *Bam*HI-*Pst*I fragment from pUCD800 subcloned into pSK-oriT. Chemiluminescent detection of biotinylated probes was performed according to the Phototope-Star detection protocol (New England Biolabs, Schwalbach, Germany).

The authenticity of the mutants was also verified by immunoblot analysis on whole-cell extracts with the anti-Omp10 MAb A68/07G11/C10 and anti-Omp19 MAb.

All *Brucella* transconjugants analyzed were checked for purity, species, and biovar characterization by standard procedures by J.-M. Verger and M. Grayon (3).

Indirect enzyme-linked immunosorbent assay on whole *Brucella* cells was performed as described previously (6) with anti-Omp10 MAb to detect Omp10 and with anti-peptidoglycan MAb 3D6 to assess outer membrane integrity (11).

For electron scanning microscopy, freshly grown *Brucella* cells were suspended in phosphate-buffered saline (PBS) to an OD of 1 at 590 nm and killed by addition of gentamicin (50 μg/ml) and sodium azide (0.1%). Cells were fixed with glutaraldehyde (2%) for 15 min, and 1.5 ml was transferred on a 0.2-μm-pore-size cellulose acetate filter (Sartorius). Samples were dried, coated to a thickness of 20 nm with a Balzers DCM010 gold sputter coater, and viewed with a Philips XL20 scanning electron microscope.

Susceptibility assays. *Brucella* strains were tested for sensitivity to bovine serum essentially as described by Corbeil et al. (12). Sera were collected from blood of two naive cattle and stored at −80°C. The log of the number of CFU per milliliter of fresh serum was subtracted from the log number of CFU per milliliter of heated serum. Results were expressed as the log of the number of bacteria killed by 1 ml of serum (referred to herein as the "log killed"). Bacterial survival after a controlled exposure to polymyxin B (7,870 U/mg; Sigma-Aldrich Chemie, Steinheim, Germany) was assayed essentially as described by Sola-Landa et al. (40). *B. melitensis* rough mutant B3B2 was used as a control strain sensitive to polymyxin B (25). Briefly, serial dilutions of polymyxin B prepared in 1 mM HEPES (pH 8.0) were made in 96-well microtiter-type plates. Bacteria resuspended at around 2×10^4 CFU/ml were dispensed into triplicate rows, and plates were incubated for 1 h at 37°C. Viable counts were performed by spreading 20 μl from each well onto 2YT agar. Results were expressed as the percentage of survival with respect to that of controls incubated without the peptide. Data presented are means of triplicate rows and are representative of four experiments. MICs of detergents (sodium dodecyl sulfate, sodium deoxycholate, and *N*-lauroyl-*N*-methylglycine [Sarkosyl] [all from Sigma-Aldrich]) were determined in 2YT plates. The sensitivities of *Brucella* strains to killing by H_2O_2 as well as by antibiotics (penicillin G, ampicillin, tetracycline, erythromycin, and rifampin [bioDiscs], Bio-Mérieux, Marcy l'Etoile, France) were evaluated by a disk sensitivity assay as described by Elzer et al. (17).

Cell culture and bacterial infection. Survival of *Brucella* strains was evaluated in an immortalized cell line of bovine peritoneal macrophages (41) and in epitheloid human HeLa cells by the procedure described by Delrue et al. (14). Briefly, *Brucella* strains were grown to log phase for 18 h in 2YT broth in the presence of the appropriate antibiotics and added to cells at a multiplicity of infection of 200 to 300 in cell culture medium. Culture plates were centrifuged for 5 min (for macrophages) or 10 min (for HeLa cells) at 1,000 rpm in a Jouan centrifuge at room temperature and placed in a 5% CO_2 atmosphere at 37°C. After 1 h, wells were washed and further incubated with culture medium supplemented with 50 μg of gentamicin (Life Technologies) per ml to kill the remaining extracellular brucellae. The number of intracellular viable brucellae was determined at 2, 24, and 48 h postinfection. The data presented are means of four to six culture wells and representative of three experiments.

Survival in the mouse model. Groups of 8 (first assay)- or 10 (second assay)-week-old female BALB/c mice were inoculated intraperitoneally with 0.2 ml of a suspension containing around 10^4 CFU of each bacterial strain harvested with PBS from a 24-h TSAYE slope (exact doses were retrospectively assessed). At appropriate intervals postinoculation (p.i.), five mice from each treatment group were sacrificed for blood and spleen collection. The presence of O-antigen antibody in the mouse sera was determined by enzyme-linked immunosorbent assay. Spleens were weighed, and bacterial survival was determined following homogenization of the mouse spleens in 5 ml of PBS (first assay) or 2 ml of distilled water (second assay) with a stomacher 80 homogenizer. Serial dilutions of the spleen homogenates were plated in triplicate on TSAYE to determine bacterial counts. For the first assay, the detection limit was 50 CFU per spleen. For the second assay, the whole-spleen homogenates from mice infected with strain 544D10.1 and recovered at 8 and 14 weeks p.i. were plated in order to reduce the detection limit from 20 to 1 CFU per spleen. The number of CFU per spleen was expressed as the log number of CFU per spleen. The maintenance of the phenotypic and genetic markers was checked throughout these experiments. The 544D19 and 544D10 isolates tested showed the same phenotypic properties and immunoblot patterns as the inoculum, indicating that they were stable.

Statistical analysis. To determine the significance of differences observed in our experiments, pairwise comparisons were performed by Scheffé tests, after a two-way analysis of variance providing the residual mean square estimate with the highest available degree of freedom number (13).

RESULTS

Construction of *omp10* and *omp19* mutants. The majority of the *omp19* coding sequence was removed from the chromosome of virulent *B. abortus* 544 Nal^r by the gene replacement strategy originally described by Halling et al. (26). Among 278 Nal^r Kan^r colonies isolated following introduction of the pD193 deletion vector into *B. abortus* 544, only six were Amp^s. The failure of these six isolates to express Omp19 was confirmed by immunoblotting with an anti-Omp19 MAb. Two Kan^r Amp^s colonies were chosen for further study and given the designation 544D19.1 and 544D19.2. Southern blot analysis of these two putative *omp19* mutants confirmed gene replacement (data not shown). Following *Hin*dIII digestion, the 3.8-kb band characteristic of the presence of *omp19* in the 544 parent DNA (48) was replaced by two bands of 3.1 and 1.6 kb in both 544D19 mutant DNAs, consistent with *omp19* replacement by *kan* containing an *Hin*dIII site. The *kan* probe did not hybridize to the 544 DNA but did hybridize to the same-size fragments recognized by the *omp19* probe from both 544D19 mutants.

Cloning of larger fragments upstream and downstream of *omp19* into pD193 allowed us to isolate *omp19* mutants (probably by increasing the frequency of crossovers), although with a low efficiency. To increase the efficiency of deletion mutant isolation upon conjugal transfer of replacement vector, we used the *B. subtilis sacB* gene as a counterselectable marker in *B. abortus* 544. Therefore, plasmid pDS10 was constructed by introducing the *sacB* marker into plasmid pD103, designed for *omp10* gene replacement. All the Nal^r Kan^r transconjugants selected in the presence of sucrose were Amp^s. Wild-type 544 and two Amp^s Suc^r clones were analyzed by Southern blotting. Due to an *Hin*dIII internal site in the *omp10* probe, it hybridized with two bands of 4.8 and 4.5 kb in the 544 DNA (48). The 4.5-kb band was replaced by a 1-kb band in the Amp^s Suc^r clones, consistent with replacement of *omp10* coding sequence by *kan*. The *kan* probe did not hybridize to 544 DNA but did hybridize to 4.5- and 1-kb fragments in Amp^s Suc^r clones. These data confirm that the allelic exchange had occurred, resulting in the *omp10* gene being replaced by the *kan* cassette. Western blot analysis with an anti-Omp10 MAb confirmed that Omp10 was not expressed in the Amp^s Suc^r clones. Two Nal^r Kan^r Amp^s Suc^r clones were chosen for further study and named 544D10.1 and 544D10.2. Counterselection with *sacB* was also used successfully for the deletion of *omp10* in other *Brucella* strains or species: *B. abortus* vaccine strain S19, *B melitensis* 16 M, and *Brucella ovis* Reo 198.

omp10 and *omp19* are not essential genes for *Brucella* survival in vitro. This was shown by the deletion of the genes from several brucellae. In addition, deletion of the *omp10* gene or *omp19* gene had no detectable effect on conventional species and biovar phenotypic properties, and oxidative metabolic patterns (data not shown) (3). Omp10 was not shown to be involved in Tb, Iz, Wb, or R/C brucella phage adhesion or entry, since phage susceptibility patterns of the parent *B. abortus*, *B. melitensis*, *Brucella suis*, *B. ovis*, and the *omp10* mutants are identical. Omp19 is also not necessary to infection by the first

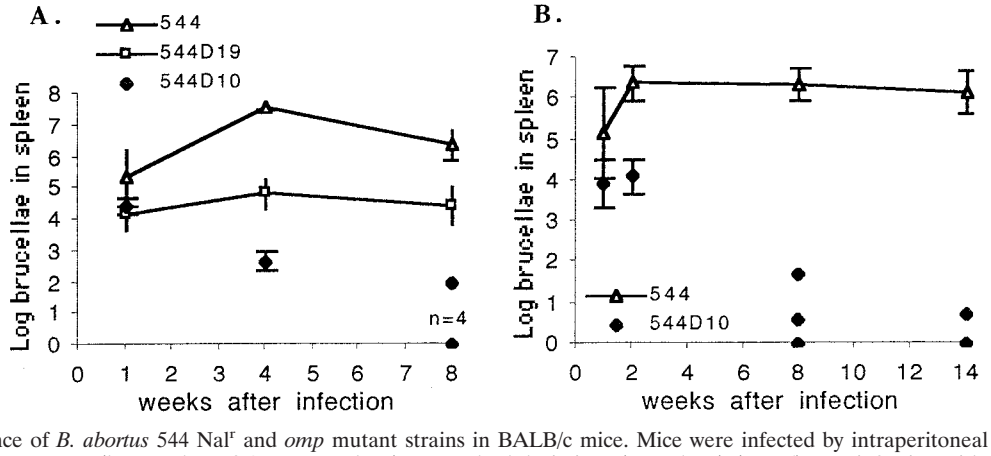

FIG. 1. Virulence of *B. abortus* 544 Nal^r and *omp* mutant strains in BALB/c mice. Mice were infected by intraperitoneal injection with 10^4 brucellae. Values are means (log number of CFU per spleen) ± standard deviations (error bars) (n = 5). For infection with strain 554D10.1 at 8 and 14 weeks p.i., individual results are shown. (A) First infection assay with 544 Nal^r, 544D19.1, and 544D10.1. (B) Second infection assay with 544 Nal^r and 554D10.1 (n = 4; no bacteria detected in four mice).

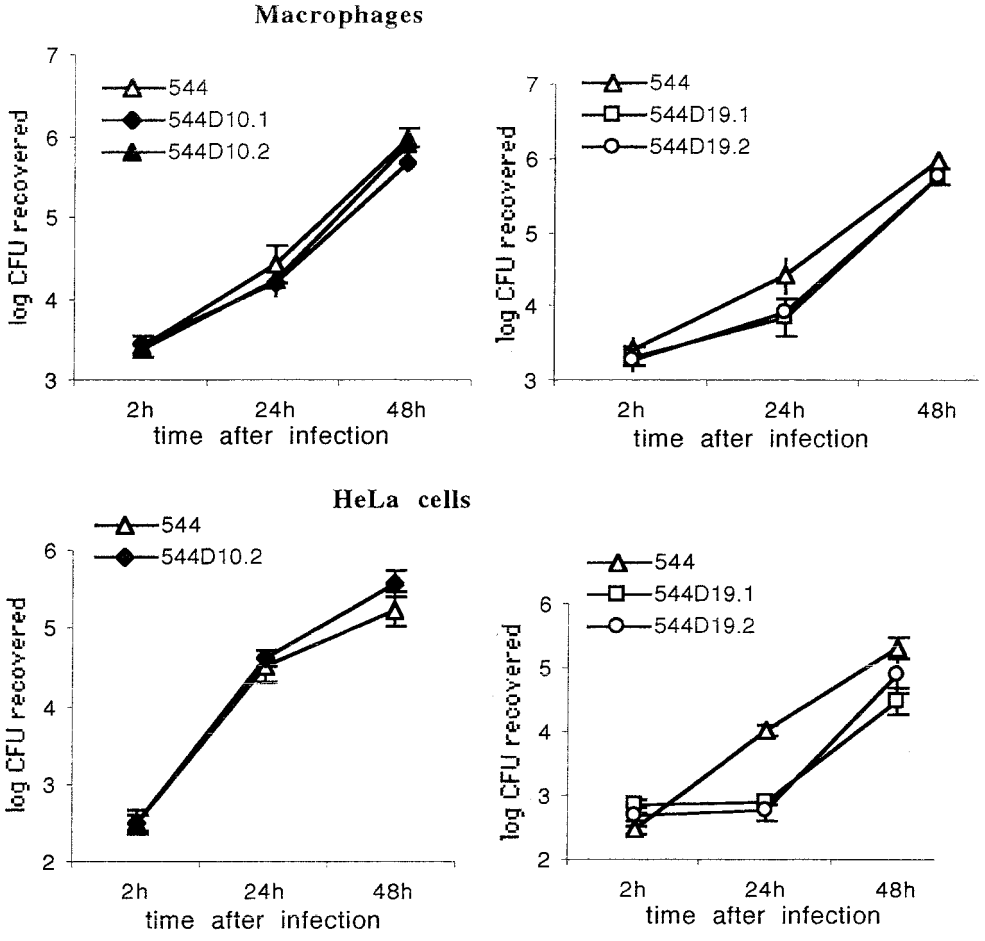

FIG. 2. Intracellular replication of *B. abortus* 544 Nal^r, 544D19.1, 544D19.2, 544D10.1, and 544D10.2 in bovine macrophages or HeLa cells. The data presented are the results of a representative experiment and are means ± standard deviations (error bars) of plate counts from four to six culture wells.

three of these brucella phages since the *B. abortus omp19* mutant remains susceptible to these phages.

Survival of the *omp* mutants in the mouse model. We compared the numbers of bacteria in the spleens of mice infected with 10^4 CFU of 544D10.1, 544D19.1, or 544 Nal^r. Growth of 544D19.1 and that of 544 were parallel over the 8-week period, but mean numbers of bacteria in the spleens of 544D19.1-infected mice were always significantly lower ($P = 0.05$ at week 1 and $P = 0.001$ at weeks 4 and 8) than the number of bacteria in the spleens of 544-infected mice (Fig. 1A). In mice infected with 544D10.1, the number of bacteria decreased at 4 weeks p.i. At 8 weeks p.i., no bacteria (detection limit, 50 CFU) were detected in the spleens of four of the five mice infected with 544D10.1, and only 10^2 CFU per spleen was detected in the spleen of the remaining mouse. In contrast, mean counts in spleens from mice infected with 544 were higher than 10^5 CFU during the entire 8-week period. The 544D10 and 544D19 mutants colonized the liver (detection limit, 50 CFU) but almost disappeared from this organ by 4 weeks, in contrast to 544, which persisted at about 4 logs for the 8-week period. These results show a slight attenuation of the *omp19* mutant in the mouse.

In order to precisely determine *omp10* mutant clearance from BALB/c mice, we performed a second infection assay with strain 544D10.1 or 544 Nal^r, with a lowered detection limit of 1 organism per spleen (Fig. 1B). The 544 Nal^r parent strain quickly grew in spleens and reached high numbers that persisted over the 14-week period. Eight weeks after infection with 544D10.1, one of the mice was free of brucellae, and only between 1 and 47 CFU per spleen was detected in the other four mice. At 14 weeks p.i., five bacteria were detected in the spleen of only one of five mice infected with this strain. Serologic data confirmed that the mice had effectively been infected by *Brucella* even though no brucellae were recovered from their spleens. These data confirmed the results of the first assay and established that 544D10.1 was attenuated in the mouse.

The experiments described in the following sections were designed to determine the effect of the *omp* mutations to several host killing mechanisms.

Survival of the *omp* mutants in cells. The ability of *omp* mutants to enter cells and to replicate intracellularly was studied in professional and nonprofessional phagocytes (Fig. 2). The parent *B. abortus* 544 strain replicated within bovine macrophages and HeLa cells. No significant difference in uptake and intracellular growth in both cell types between the *omp10* mutants and their parent strain was seen.

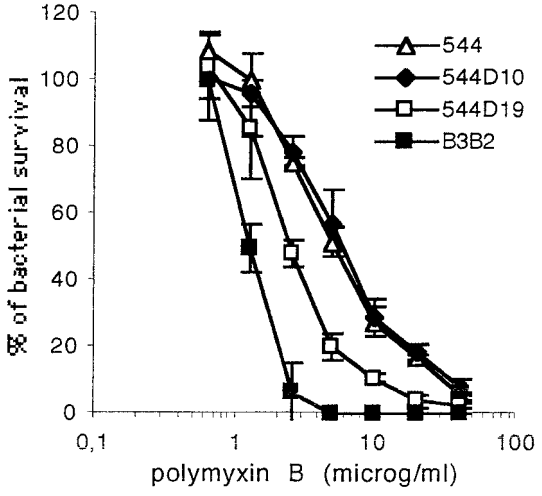

FIG. 3. Bactericidal effect of polymyxin B on *B. abortus* 544 Nal^r, 544D19.1, 544D10.1, and B3B2. The data presented are the results of a representative experiment and are means ± standard deviations (error bars) of plate counts from three wells. Results are expressed as percentages of the brucellae surviving in wells incubated in the absence of the peptide.

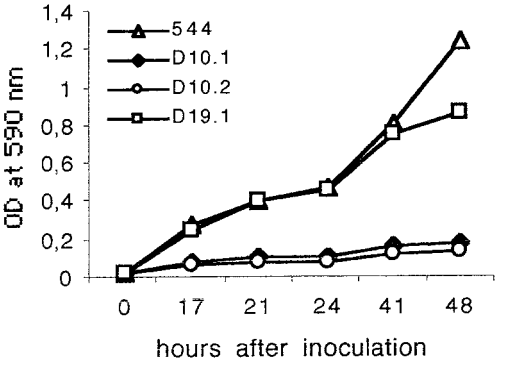

FIG. 4. Growth of *B. abortus* 544 Nal^r, 544D19.1, 544D10.1, and 544D10.2 in minimal medium. Cultures were inoculated to identical ODs from fresh cultures (0.03 at 590 nm), and growth was monitored by recording the ODs of the cultures at 590 nm at different time points during 48 h. These data are representative of three independent assays.

The *omp19* mutants replicate to levels close to those of 544 Nal^r in bovine macrophages. However, in HeLa cells *omp19* mutants replicate at a lower rate than do 544 cells during the first 24 h ($P < 0.001$). After 24 h, the replication rate increased and paralleled that of 544.

Physiological and morphological characterization of *omp* mutants. Compared to other gram-negative bacteria, *Brucella* outer membrane is resistant to bactericidal polycations such as polymyxin B. To evaluate the effect of the loss of Omp10 or Omp19 on *B. abortus* outer membrane properties, we first tested *omp* mutant viability after controlled exposure to polymyxin B. Omp10 mutants showed no increase in sensitivity to polymyxin B (Fig. 3). However, the survival of the *omp19* mutant 544D19.1 was reduced relative to that of 544 Nal^r over a polymyxin B concentration range of 2.5 to 20 μg/ml ($P < 0.001$). In addition, MIC assays showed that *omp10* and *omp19* mutants were more sensitive to sodium deoxycholate (1.5 times and 3 times more, respectively) than the parent strain, suggesting altered outer membrane properties. Sensitivities of mutant and parent strains to sodium dodecyl sulfate, Sarkosyl, and the five tested antibiotics were not significantly different.

In vitro exposure revealed no appreciable increase in the sensitivity of the *omp* mutants to H_2O_2.

Mutant and parent strains have similar carbon substrate utilization patterns. The mutants showed no defect in the ability to grow in rich media (tryptic soy broth or 2YT broth). In contrast *omp10* mutants were unable to grow in minimal medium independently of the addition of amino acids or nitrogenous bases (Fig. 4).

Mutation of Omp10 or Omp19 had no dramatic effect on the morphology of *B. abortus* 544 cells as determined by scanning electron microscopy, except that a very small proportion of 544D19 cells have a branched aspect or an irregular shape with swelling (data not shown).

DISCUSSION

The outer membrane lipoproteins Omp10 and Omp19 could constitute a new family of Omps specifically encountered in the *Rhizobiaceae*. No function has been assigned to these proteins, and analysis of the *B. melitensis* and *B. suis* genomic sequences surrounding the *omp10* and *omp19* genes has not provided more information about the function of these two lipoproteins.

Using a murine model, we found that deletion of the *omp10* gene from *B. abortus* 544 dramatically reduces bacterial virulence. Over the 14-week study period, mice clear the mutant 544D10 more rapidly than the parent strain and display almost no splenomegaly. Since significant attenuation is observed by 2 weeks, *omp10* mutant replication is probably hampered during the intracellular stage in phagocytes. The *omp10* mutants showed no reduced resistance to polycationic peptide or to the reactive oxidative agent H_2O_2, two in vitro mimics of host intracellular defenses.

Since brucellae enter and replicate within professional and nonprofessional phagocytic cells, growth in both types of cells is used as a model system for measuring the attenuation of *Brucella* mutant strains. By using a bovine macrophage cell line and HeLa cells, no in vitro intracellular attenuation of the *omp10* mutant was detected. Whether these discrepancies between in vivo and in vitro results are due to differences between mice and human or bovine cells remains to be determined. In addition, reduced *omp10* mutant survival in mice is probably not attributable to enhanced extracellular killing by complement, since no increase in complement-mediated lysis was observed for this mutant (data not shown). The only detected effect of *omp10* mutation on outer membrane properties was a 1.5-fold increase in sensitivity to sodium deoxycholate.

The *omp10* mutant exhibited a marked growth defect in minimal medium, indicating dependence for growth on an unidentified compound absent from this medium (that does not seem to be secreted by the parent strain and made available by diffusion). If this dependence is relevant to the mutant attenuation in the mouse, availability of this compound in the *Brucella* replication niche would be a limiting factor for *Brucella* infection in mice.

The results obtained with mice suggest that *omp10* mutant

virulence should be further evaluated in the natural bovine host. In addition, the efficacy of *omp10* mutant as a live vaccine against *B. abortus* should be investigated.

The sequence of the *omp10* 3′ region was determined. It contains, 138 bp downstream of the *omp10* stop codon, a coding sequence for a homologue to the HemH ferrochelatase, the final enzyme of the heme biosynthetic pathway. Although we cannot definitely preclude a polar effect of *omp10* mutation on *hemH* expression, *omp10* mutants exhibited none of the phenotypes associated with *hemH* mutants in *B. abortus* and in other gram-negative bacteria: auxotrophy for hemin and brownish red color of colonies (2, 20, 21, 52). In addition, a putative transcription terminator is present 18 bases downstream from the *omp10* stop codon (43). Attempts to complement the *omp10* mutant with a copy of the *omp10* gene cloned on the pBBR1MCS plasmid vector under the control of its own promoter failed, probably because of the overexpression of Omp10 in this strain.

Mice remained infected, but at a significantly lower level, with the *omp19* mutant 544D19 over an 8-week period and exhibited almost no splenomegaly. The *omp19* mutants showed a lower growth rate, at least in HeLa cells, during the first 24 h of infection, suggesting that they are more sensitive to the conditions within phagosomes before adapting to intracellular conditions and residency in an intracellular replication compartment. Disruption of the gene encoding Omp19 (referred to as an 18-kDa lipoprotein by these researchers) in vaccine strain *B. abortus* RB51 had no influence on the survival of this strain in mice (47), indicating that loss of Omp19 does not aggravate the attenuation of this rough strain.

Omp19 mutants exhibited altered outer membrane properties, as indicated by their reduced resistance to deoxycholate and polymyxin B. The low number of negatively charged groups in the *Brucella* LPS would account for the reduced affinity of the *Brucella* outer membrane for cationic peptides such as polymyxin B (33). The results of Freer et al. indicate that the involvement of *Brucella* outer membrane proteins in polymyxin B sensitivity "cannot be excluded, although their direct participation is unlikely" (19). Moreover, brucellae are sensitive to bovine complement-mediated lysis via the classical pathway (12). This sensitivity is a factor of extracellular killing within the host. Preliminary results suggest that the *omp19* mutant exhibited an increased sensitivity to bovine serum complement compared to that of the parent 544 strain (1.8 log killed compared to 1.1 log killed).

All together, these results suggest an indirect effect of Omp19 deletion on interaction between polymyxin B and *Brucella* outer membrane, either at the initial interaction step or beyond this step. Freer et al. (19) indeed also showed that non-LPS outer membrane molecules participate in the arrangement of the LPS lattice. It is possible that the absence of Omp19 modifies interactions between outer membrane components which could disorganize this lattice. Enhanced sensitivities to deoxycholate and bovine complement-mediated lysis are also consistent with this hypothetical fragility of the *omp19* mutant outer membrane. It would be interesting to evaluate the effect of *omp19* mutation on the naturally rough *B. ovis* strain outer membrane properties to eliminate the contribution of the O-chain.

The slight but significant attenuation of 544D19 mutant in mice could be attributed to altered outer membrane properties of this mutant which would affect both its extracellular and intracellular survival.

Further studies are necessary to establish Omp10 and Omp19 function in *Brucella* growth and pathogenesis.

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